Journal of Solid-Phase Biochemistry, Vol. 2, No. 2, 1977

KINETICS OF INACTIVATION AND EFFECTS OF STABILIZATION OF CHLOROPLASTS

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Accepted June 9, 1977

The kinetics of inactivation of the electron transport chain of isolated chloroplasts was investigated. Methods for the stabilization of chloroplasts were developed. The mean operating time of the system was increased by a factor of about 200.

INTRODUCTION

Problems that arise in studies of the inactivation of biological structures are of interest from various points of view. Loss of functional activity of protein catalysts and biopolymer structures is the major concern of modern molecular gerontology. On the other hand, the use of unique properties of biopolymer structures in various fields of biological engineering attracts the increasing interest of scientists (1). In this connection, the study of cellular and subcellular structures capable of realization of complex synthetic sequences is of importance.

The mechanism of photosynthesis and patterns governing inactivation of photosynthesizing systems have attracted a good deal of attention during recent years (2,3,9,10). Our interest in the inactivation of isolated chloroplasts stems in large part from their potential utility in systems for the biophotolysis of water (4-8) and photohydrogen production (11).

It is clear from general considerations that the stability of chloroplasts can be modified by modifying their environment, the media in which they occur.

This work has been conducted in order to obtain quantitative information about the effects of media on the stability of the electron transport chain in isolated chloroplasts.

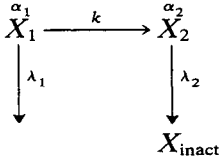
KINETIC MODEL

The determination of quantitative parameters providing an adequate description of systems stability is the principal problem in the quantitative

analysis of inactivation of so complex a system, particularly from the physicochemical point of view in systems such as isolated chloroplasts. In earlier papers (9,19) we described a kinetic model for inactivation of chloroplasts based on the following assumptions.

1. Inactivation of chloroplasts proceeds via sequential formation of various structured forms in organelles with different functional activities.
2. Each form has individual stability which can be described by a time-independent frequency of inactivation events. Kinetically, this parameter represents the inactivation rate constant of a given form. In terms of the theory of reliability (12), each form is characterized by time-independent failure intensity. This assumption appears to be a rather realistic one when considered within the framework of the theory of reliability of enzymes and multienzyme systems (13).

Analysis of the kinetic model based on these assumptions and its comparison with the experimental data on the kinetics of inactivation of isolated chloroplasts from pea have led us to conclude that inactivation of chloroplasts involves at least two forms which differ in stability and activity:



where X_1 and X_2 are structurally and functionally different forms of chloroplasts, X_{inact} is a denaturated, inactive form, α_1 and α_2 are partial activities of forms X_1 and X_2, respectively, k is the effective rate constant of transformation of form X_1 to X_2, and λ_1 and λ_2 are failure intensities of the respective forms (or inactivation rate constants, in terms of chemical kinetics).

The values of parameters $k+\lambda_1$ and λ_2 can be determined from the experimental data using the kinetic model adopted. This can be done by linearization of inactivation curves in semilogarithmic coordinates (10). The process with frequency λ_2 is irreversible inactivation of form X_2. This is the fastest reaction involved in inactivation of chloroplasts. The parameter λ_2 (inactivation rate constant, failure intensity of form X_2) thus provides a convenient quantitative characteristic of the overall stability of isolated chloroplasts.

METHODS

Chloroplasts were isolated from pea leaves ("Moskovskii-73") using the procedure previously described (14). Buffer for isolation (12 ml, pH 7.8) containing sodium phosphate (40 mM), NaCl (10 mM), bovine serum albumin (BSA, 0.1%), and glycerol or sucrose (0.4 M) was added to the pea leaves (5 g). The mixture was ground, filtered through four layers of nylon sheets, and centrifuged at $500 \times g$ for 3 min. The supernatant was then centrifuged at $2500 \times g$ for 6 min. Chloroplasts thus obtained were washed by suspending them in buffer for isolation (15 ml) and then centrifuging once more at $5000 \times g$ for 8 min. All steps of the isolation process were carried out at 4°C. Washed chloroplasts were then suspended in the desired medium and incubated at a constant temperature. Concentrations of chloroplasts under the incubation conditions corresponded to 0.12 mg chlorophyll/ml.

Bovine serum albumin from Koch-Light was used. Glycerol and components of buffer solutions were of spectroscopically pure grade.

Isolated chloroplasts were incubated under standard conditions in the dark in media of varying composition. Photosynthetic activity assays were carried out using a model reaction. One of the experiments was performed in the presence of an antibiotic (chloramphenicol, 1 mg/ml) to show that bacterial contamination does not affect inactivation of chloroplasts.

Photosynthetic activity assays were made by measuring rates of photoreduction of ferricyanide anions using spectrophotometer Hitachi-124. The standard conditions for activity assays were pH 7.4, phosphate (40 mM), NaCl (10 mM), chlorophyll (14 μg/ml), $K_3Fe(CN)_6$ (0.7 mM). In experiments with glycerol, the latter was added in a concentration of 8%. Photoreduction was carried out in spectrophotometric cells. Solutions were continuously stirred and maintained at the constant temperature of 25 ± 0.1°C. A 100-W white-light halogen lamp in combination with a condenser system was used as light source. Concentration of ferricyanide was monitored by measuring absorption of samples at 420 nm (ferricyanide has an ε of $10^3\ M^{-1}\ cm^{-1}$ at 420 nm). Typical exposure times were 5–10 min.

RESULTS

Kinetics of Inactivation of Chloroplasts as Depending on Glycerol Concentration.

Isolated chloroplasts were incubated at various concentrations of glycerol and then assayed for activity in the Hill reaction. Figure 1 shows

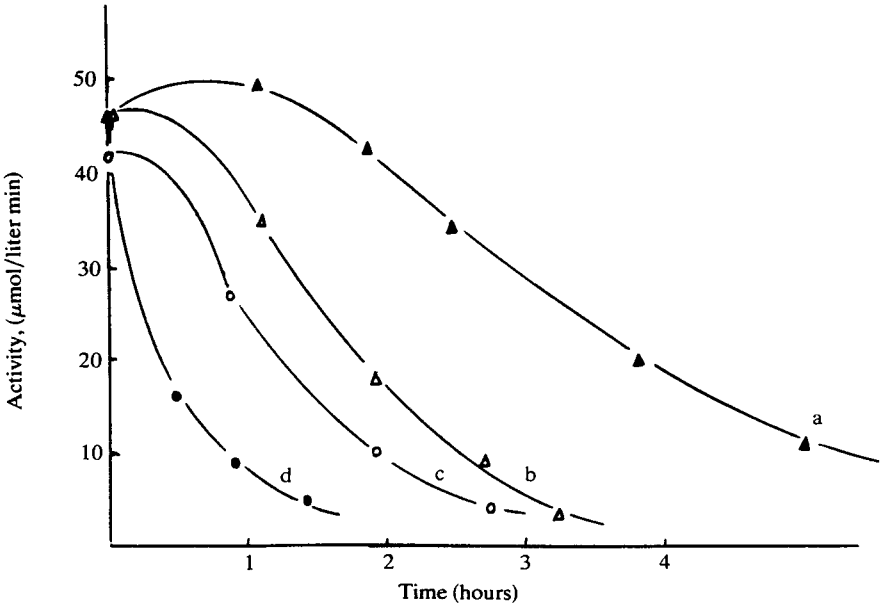


FIG. 1. Kinetic curves of inactivation of chloroplasts at various concentrations of glycerol in the incubation medium (wt %): a, 3.7; b, 16.4; c, 40; d, 70. Incubation conditions: pH 7.8, 35°C, 10 mM NaCl, 40 mM phosphate.

typical kinetic curves of inactivation of chloroplasts under such conditions. Linearization of the experimental curves according to the procedure described earlier (10) gives a parameter λ_2 characterizing the stability of chloroplasts. Stability of chloroplasts as a function of glycerol concentration features an extremum (Fig. 1). High glycerol concentrations do not affect the initial activity of chloroplasts, but significantly accelerate their inactivation.

Kinetics of Inactivation of Chloroplasts as Depending on Bovine Serum Albumin Concentration.

Inert proteins, first of all, BSA, often act on biological organelles as stabilizing admixtures (15,16). We have studied the kinetics of inactivation of chloroplasts as depending on the concentration of BSA in detail. A typical kinetic curve is shown in Fig. 2. Admixtures of albumin stabilize chloroplasts to a considerable extent. The effect, however, is of extremal nature, like that of glycerol. Figure 3 illustrates the dependence of the parameter λ_2 on albumin concentration (two curves correspond to two

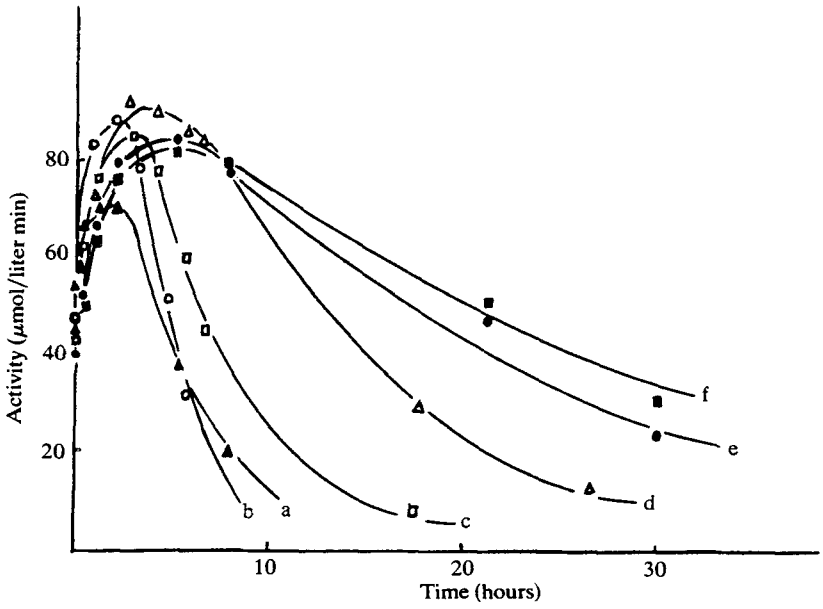


FIG. 2. Kinetic curves of inactivation of chloroplasts at various concentrations of BSA in the system (M): a, 0; b, 10^{-5}; c, 3×10^{-5}; d, 6×10^{-5}; e, 10^{-4}; f, 10^{-3}. Incubation conditions: glycerol 40%, phosphate 40 mM, NaCl 10 mM, EDTA 10 mM, pH 7.8, 35°C.

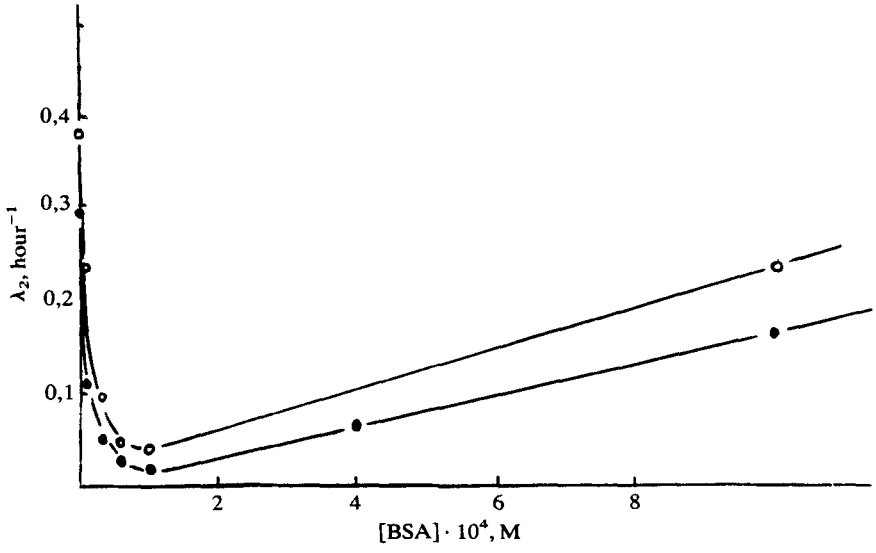


FIG. 3. Effective inactivation rate constant of chloroplasts as a function of BSA concentration in the medium. For conditions see Fig. 2.

independent experiments). The initial stabilities of various samples of chloroplasts are somewhat different (the inactivation rate constant values differ by about 25%). The shapes of the two curves are, however, very much the same. The maximum stability of chloroplasts is observed at about a 0.1 mM concentration of albumin.

Kinetics of Inactivation of Chloroplasts as Depending on Proton Concentration.

Proton concentration is an important factor determining the stability of biopolymer structures. We have studied pH profiles of inactivation parameters for chloroplasts stabilized by the presence of optimum concentrations of glycerol and albumin. For comparision, inactivation of chloroplasts in the initial medium containing 3.7% of glycerol was studied at various levels of pH. The experimental λ_2 values as functions of pH are shown in Fig. 4 for all three cases. The pH profiles of λ_2 represent inverted

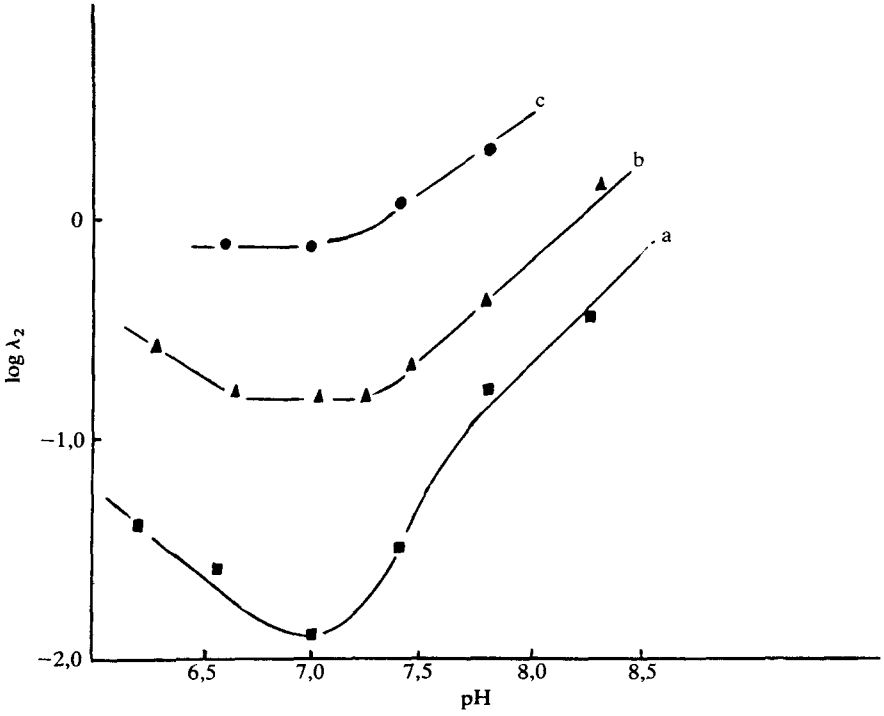


FIG. 4. Effective inactivation rate constant of chloroplasts as a function of pH. a, 3.7% glycerol; b, 40% glycerol; c, 40% glycerol + p.1 mM of BSA. For conditions see Figs. 1 and 2.

bell-shaped curves. The maximum stability is observed at a pH of about 7.0.

Our study of the kinetics of inactivation of isolated chloroplasts *in vitro* shows that processes responsible for the loss of the photosynthetic activity of chloroplasts are very sensitive to external conditions, which are determined by physicochemical properties of the medium. A characteristic feature of all kinetic parameter versus medium composition dependences studied is the presence of extrema (cf. Figs. 1, 3, and 4). One particular medium component may have both stabilizing and destabilizing action depending on its concentration. This observation is of practical importance for it emphasizes the necessity to maintain the composition of the medium accurately in experiments on stabilization of isolated chloroplasts *in vitro*. According to the results obtained in this work, the optimum conditions for storing chloroplasts from the point of view of electron transport chain stability (without the use of low temperatures) are the following: pH 7.0, phosphate 40 mM, EDTA 10 mM, BSA 0.1 mM, and glycerol 40%.

Time intervals during which chloroplasts retain satisfactory activity can be estimated as follows. The theory of reliability of physical systems, the application of which to biological problems is described in earlier articles (11–13), suggests that the mean time to failure is a quantitative characteristic of operating time. This is given by

$$T_0 = \int_0^\infty \frac{A(t)}{A_0} \mathrm{d}t \tag{2}$$

where $A(t)$ is the time-dependent activity of a system and A_0 is the initial activity. For chloroplasts operating under optimum conditions, Eq. (2) gives a T_0 value of 360 h (35°C), while the initial system is characterized by a T_0 of 2 h (35°C). Thus, investigation of the effects of stabilizing admixtures on the kinetics of inactivation of chloroplasts, and optimization of medium composition have allowed us to increase the mean operating time of the system by a factor of about 200.

The basic problem of inactivation of biopolymer systems is that of the physicochemical nature of the process leading to inactivation. This problem remains unsolved. A very general consideration pointing to some physicochemical transformations of biomembranes as causes of inactivation can only be put forth as far as isolated chloroplasts are concerned. The fact that various factors, such as solvent, protein polyelectrolytes, and protons, influence inactivation of chloroplasts shows that the process can be regulated. We believe that systematic investigations of such effects will provide both fundamental information about the nature of "aging" of

organelles and the means of considerable stabilization of organelles in the operating state.

ACKNOWLEDGMENT

The authors are indebted to Professor I. V. Berezin for interesting and valuable discussions.

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